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T lymphocyte lines producing or vaccinating against autoimmune encephalomyelitis (EAE). Functional activation induces peanut agglutinin receptors and accumulation in the brain and thymus of line cells*

We studied lines of rat T cells, specifically reactive against myelin basic protein (BP), that were functional in mediating autoimmune encephalomyelitis or in vaccinating rats against induction of active EAE. Herein we report that these functions depended on activation of the cells by incubation with BP or with a T cell mitogen prior to inoculation into recipient rats. Activation was accompanied by the exposure of membrane-binding sites specific for the lectin peanut agglutinin. Accumulation of activated line cells in the central nervous system and thymus gland was observed.

1 Introduction

We have succeeded in isolating and growing Lewis rat T lymphocyte cell lines specifically reactive against the basic protein of myelin (BP) [1]. These cell lines were found to be functional and mediated clinical and pathological signs of experimental autoimmune encephalomyelitis (EAE) upon i.v. inoculation into syngeneic recipient rats. Furthermore, attenuated cells of the anti-BP lines could be used to vaccinate rats against the development of active EAE subsequent to immunization against BP in complete Freund's adjuvant (CFA) [2, 3]. Herein we report that the realization of these functions by line

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Abbreviations: BP: Myelin basic protein CFA: Complete Freund's adjuvant CNS: Central nervous system Con A: Concanavalin A EAE: Experimental autoimmune encephalomyelitis PBS: Phosphate-buffered saline PNA: Peanut agglutinin PPD: Purified protein derivative of mycobacteria IL2: Interleukin 2 (T cell growth factor) LC: Line cells

cells (LC) required their activation *in vitro* before inoculation into recipient rats. Nonactivated anti-BP LC could neither cause EAE nor prevent disease. In addition to acquisition of a functional program, activation was associated with changes in the cell membrane detected by the exposure of receptors specific for the lectin peanut agglutinin (PNA). Moreover, accumulation of some cells in the brain just before onset of EAE was a unique characteristic of activated anti-BP LC. The thymus trapped a minority of LC of any specificity, provided that the cells had been activated by incubation with specific antigen before injection. The majority of all injected cells, activated or not, accumulated in the liver and in the spleen. Thus, the entry and persistence of LC in various organs is influenced by state of the cells as well as by the presence of specific target antigen in the organ.

2 Materials and methods

2.1 Animals and antigens

Rats, antigens, active immunization to guinea pig BP and proliferative responses were as described previously [1-4].

2.2 Selection and propagation of antigen-specific T cell lines

Z1a LC were selected from a population of BP/CFA-primed lymph node cells for their response to BP, and Z1c line cells

for their response to purified protein derivative of tuberculin (PPD), as previously described [1]. Cell lines were maintained in medium enriched with interleukin 2 (IL2, T cell growth factor) in the absence of specific antigen or accessory cells, as described [1, 4].

2.3 Activation and transfer of LC

LC were activated by restimulation *in vitro* with either the BP or PPD antigens or the mitogen concanavalin A (Con A). LC (2×10^5 /ml) were stimulated with either antigen (10 µg/ml) or Con A (1.25 µg/ml) in the presence of syngeneic irradiated (1500 rds) normal thymus cells (15×10^6 /ml) in tissue culture plastic dishes (10 ml/dish) in a proliferation medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 1 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, gentamycin (40 µg/ml) and 1% fresh autologous rat serum. The cultures were incubated for 72 h at 37°C in humidified air plus 7.5% CO₂. The cells were then collected and washed twice, and the indicated numbers of lymphoblasts were injected i.v. into normal syngeneic recipients. The rats were observed for development of EAE diagnosed clinically by overt paralysis at least of the hind limbs and pathologically by perivascular mononuclear cell infiltrates seen on histological examinations [5].

2.4 Lectin binding and separation of cells

The lectin agglutinins, PNA, soy bean, erythrina, wheat germ, Con A, ricin and *louis tetragonolobus* were prepared and labeled with fluorescein [6]. LC were studied for lectin binding either 72 h after activation or before activation when they had been cultured for at least 14 days in medium containing IL2. Lectin binding was detected by incubating 4×10^6 cells in 0.1 ml phosphate-buffered saline (PBS) containing 50 µg lectin for 1 h at 4°C. The cells were washed by centrifugation in PBS and studied using a fluorescence microscope with a 585-nm filter. Lectins listed as positive stained >75% of the cells, while negative lectins bound to 10% or less of the cells. The results for binding of PNA were confirmed using a fluorescence-activated cell sorter (FACS II, Becton-Dickinson Electronics, Mountain View, CA), as described [4]. The specificity of PNA binding was tested by inhibition of binding in the presence of 0.2 M galactose. LC agglutinable with PNA were separated from nonagglutinable LC according to the method of Reisner et al. [7]. Briefly, anti-BP LC were activated and 10^6 cells were incubated in 0.5 ml PBS containing 1 µg PNA (Miles Yeda, Rehovot, Israel) for 10 min at room temperature. The cells in 0.4 ml were overlaid on 10 ml of 5% bovine serum albumin in a conical centrifuge tube at room temperature for 30 min. Cells were collected from the top (PNA-nonagglutinable) and bottom (PNA-agglutinable) of the tube and these fractions were washed once by centrifugation in PBS. The pellet was incubated with 2 ml of 0.2 M D-galactose for 5 min to separate clumped cells. About 75% of the activated LC were recovered in the pellet of PNA-agglutinable cells. Rats were inoculated with 1×10^6 cells and observed for development of EAE.

2.5 ⁵¹Cr labeling of LC

LC (25×10^6) were incubated for 30 min at 37°C in 1 ml of DMEM containing 250 mCi = 9.25 GBq of Na₂⁵¹CrO₄ (Amer-

sham International, Amersham, Bucks, GB) supplemented with 5% calf serum. The cells were then washed 3 times by centrifugation and resuspended in PBS at a concentration of 5×10^6 /ml. ⁵¹Cr taken up by the LC and accumulating in various organs was counted using a gamma detector. Recipient rats were exsanguinated at various times and radioactivity was measured in 1 ml of blood and in the liver, spleen, thymus, brain and spinal cord. Results are expressed as the minimal estimated accumulation (MEA) of labeled LC per organ or per 10 ml of blood as follows:

$$\text{MEA} = \frac{(\text{cpm in organ} - \text{background cpm}) \times \text{number of cells injected}}{(\text{cpm of the cells injected} - \text{background cpm})}$$

Rats were studied individually in these experiments. About 25% of the injected cpm were recovered in the sampled organs. In preliminary experiments, whole body scan of recipient rats inoculated with anti-BP LC tagged with 111 indium oxine [8] showed no major sites of accumulation outside of the liver and spleen. Thus, the bulk of recoverable LC seemed to be in the organs which we sampled.

3 Results

3.1 Activation of LC required for EAE

In previous experiments, EAE was successfully mediated by viable, proliferating anti-BP LC that had been activated by incubation with BP before inoculation. No disease was produced by inoculating cells that had been irradiated (1500 rds; [2]), treated with mitomycin C [2] or killed by heating (65°C). The supernatant fluid of Z1a cultures also failed to mediate EAE.

Table 1. Kinetics of incubation with BP for activation of Z1a line^a

Time of incubation of Z1a cells with BP (h)	Mediation of clinical EAE			
	Incidence	Degree ^b	Mean day of onset	Mean day of recovery
None	0/5	—	—	—
0	0/5	—	—	—
6	4/5	Mild	5.4	9.9
8	3/5	Moderate	7.2	10.7
18	5/5	Severe	7.2	10.7
24	5/5	Severe	7.2	10.7
48	5/5	Severe	7.2	10.7
72	5/5	Severe	7.2	10.7

- a) Z1a LC (10×10^6) that had been propagated *in vitro* in medium containing IL2 without BP were injected i.v. before restimulation (none), or after being incubated from 0 to 72 h with BP in the presence of irradiated normal syngeneic thymus cells as accessory cells. For times of incubation of 8 h or less, 6×10^6 Z1a cells were mixed with 3×10^6 accessory cells and 200 µg BP and the mixture was inoculated immediately into recipient rats (0 time) or after incubation for 6 or 8 h. Incubation for longer periods was done as described in Sect. 2.3 and each rat received 6×10^6 lymphoblasts.
- b) Weakness of tail and hind legs was considered as mild clinical EAE, paralysis of tail and hind legs as moderate clinical EAE, and paralysis of tail, hind and front legs as severe clinical EAE.

Table 1 shows that successful activation of the Z1a line was a function of time of incubation with BP. Z1a cells that were merely mixed with BP and then injected into recipient rats (time 0) produced no EAE. Six hours of incubation led to EAE that had a prolonged latent period and was relatively weak clinically. Longer periods of incubation with BP led to a faster onset of more severe disease. Thus, mediation of EAE by anti-BP LC required their activation for a period of time and was not the result of merely injecting the cells together with BP.

3.2 Con A activates the EAE potential of LC

The above results raised the question of whether the BP antigen itself was necessary to activate the EAE potential of anti-BP LC or whether functional activation could be achieved by stimulation of the cells with a mitogen. To study this question we incubated Z1a anti-BP or Z1c anti-PPD LC with BP or with Con A and inoculated recipient rats with graded numbers of cells. The results in Table 2 indicate that Con A endowed the Z1a line with the capacity to produce EAE. Hence, it appeared that mediation of EAE was a property of a specific anti-BP line, but that activation of this potential could be achieved by nonspecific stimulation with Con A.

3.3 Activated LC required for vaccination

To learn whether activation of LC was required for expression of their ability to vaccinate against EAE, Z1a anti BP or Z1c anti-PPD LC were either activated by incubation *in vitro* for 72 h with their respective antigens or maintained in medium with IL 2. Some of the LC were then treated by irradiation or mitomycin C and inoculated i.v. into naive recipient rats. To test the ability of the LC to vaccinate against EAE, the rats were challenged 3 weeks later with an encephalitogenic injection of BP/CFA. The results are tabulated in Table 3. Control rats that had not been previously inoculated with LC and rats that had been inoculated with Z1c anti-PPD cells showed no resistance to induction of EAE. As we observed earlier [2], activated Z1a anti-BP cells, attenuated by irradiation or mitomycin C, functioned as agents of vaccination and 67-70% of the recipient rats were resistant to active EAE. However,

Table 2. Activation of LC with BP or Con A^a

Cell line	Incubation with BP				Incubation with Con A			
	No. of cells	Mean latent period of onset of EAE, day of	Mean incidence of EAE, %	Mean day of onset of recovery	No. of cells	Mean latent period of onset of EAE, day of	Mean incidence of EAE, %	Mean day of onset of recovery
Z1a	20	8.5	35	6.5	20	8.5	35	6.5
Z1c	20	8.5	35	6.5	20	8.5	35	6.5

a) Z1a LC that had been propagated *in vitro* without BP were activated by restimulation with either BP (10 µg/ml) or Con A (1.25 µg/ml) as described [1] and were injected i.v. (10 × 10⁶/rat) into normal syngeneic Lewis rats.

Table 3. Vaccination against EAE requires activated anti-BP LC^a

Cell line inoculated	Treatment	Incidence of active EAE (%)	Inhibition (%)
None	None	100 (20) ^b	-
Z1a (anti-BP)	Activated	30 ^c (30)	70
	Irradiated	33 ^c (21)	67
	Mitomycin C	100 (15)	0
	Nonactivated	100 (15)	0
Z1c (anti-PPD)	Activated	100 (10)	0
	Irradiated	100 (10)	0
	Unirradiated	100 (10)	0
	Nonactivated	100 (10)	0
	Irradiated	100 (10)	0
	Unirradiated	100 (10)	0

a) Active EAE was induced by BP/CFA in Lewis rats that had been vaccinated 3 weeks earlier by i.v. inoculation (10 × 10⁶ cells/rat) or irradiated (1500 rds) or mitomycin C-treated [2] anti-BP Z1a or anti PPD Z1c LC that were maintained *in vitro* without antigen in medium containing IL 2 (nonactivated) or were restimulated, before treatment, with the appropriate antigen in the presence of irradiated syngeneic cells (activated).

b) Numbers in parentheses refer to numbers of rats.

c) P = 0.05.

Table 4. Activated anti-BP LC become PNA⁺

Fluoresceinated lectins	LC	
	Not activated	Activated
PNA	-	+
Con A	+	+
Soy bean	+	+
Ricin	+	+
Wheat germ	+	+
Erythrina	+	+
<i>Lotus tarragonolobus</i>	-	-

a) Fluoresceinated lectins were studied for their binding to anti-BP LC that were not activated, or that had been activated by incubation with BP in the presence of accessory cells. The results obtained with PNA were confirmed using a fluorescence-activated cell sorter and showed that less than 10% of the LC were positive before activation, whereas 100% of the cells were positive after activation. Specificity was tested by inhibition of PNA binding in the presence of 0.2 M galactose.

these LC could not vaccinate if they had not been activated by incubation with BP. Thus, vaccination, similar to mediation of disease, appeared to require activation *in vitro* of anti-BP LC.

3.4 Activated LC become PNA⁺

We studied binding of fluorescein-labeled lectins to living LC to investigate whether activation involved changes in the cell membrane. Table 4 shows that of 7 lectins tested, only PNA distinguished between activated and nonactivated LC in that

activation was associated with acquisition of positive PNA binding. Thus, activation was associated with the availability of receptors specific for PNA on the cell membrane.

3.5 PNA-agglutinable activated LC mediate EAE

To investigate whether PNA agglutinability was a marker for functionally potent LC, we activated anti-BP LC by incubation with BP in the presence of irradiated accessory cells and physically separated the LC into those that were agglutinable or not agglutinable by PNA [7]. Table 5 shows that the PNA-agglutinable cells were more effective in mediating EAE than were the nonagglutinable cells.

3.6 Organ distribution of anti-BP LC

Fig. 1 shows the results of 1 of 4 experiments in which Lewis rats were inoculated i.v. with the anti-BP LC that had been activated by incubation for 3 days with BP in the presence of irradiated accessory cells. All four experiments produced similar results. The activated anti-BP LC disappeared from the blood within 1 or 2 days and a relatively large number accumulated in the liver (about 90% of the recovered counts), while a lesser number accumulated in the spleen (about 10%). On day 4, one day before the onset of EAE, there was a relatively small but highly significant accumulation of counts in the brain and thymus (about 1% each). The spinal cord (not shown) accumulated about the same number of tagged anti-BP LC as did the brain. The brains of a total of 10 rats that were examined one day before onset of paralysis, in the 4 experiments, showed an estimated accumulation of 11396 ± 2310 tagged anti-BP LC.

Fig. 2 shows the results of an experiment in which we followed the organ distribution of activated and irradiated anti-BP LC. It can be seen that these cells failed to accumulate in either the brain or the thymus and were found in the liver and spleen. Nonactivated anti-BP LC showed the same pattern of distribution (not shown). Thus, the ability of intact activated anti-BP LC to mediate EAE was correlated with the accumulation of some of the LC in the brain and thymus.

3.7 Accumulation of anti-PPD LC

To test the specificity of accumulation of LC in the brain, we tagged activated cells of the Z1c anti-PPD line with ^{51}Cr and

Table 5. Activated anti-BP EAE effector LC are agglutinable by PNA^{a)}

Group	Agglutinable (%)	Nonagglutinable (%)
Activated anti-BP LC	90	10
Irradiated anti-BP LC	10	90

a) Activated anti-BP LC were separated into fractions that were or were not agglutinated by PNA. Lewis rats were inoculated with 10^6 cells of each fraction and observed for paralysis of tail and hind legs as signs of moderate EAE.

injected rats i.v. with 5×10^6 cells. The estimated number of cells accumulating in the liver, spleen, thymus and brain is shown in Fig. 3. Activated anti-PPD cells accumulated in the liver, spleen and thymus to about the same degree as did activated anti-BP LC (see Fig. 1). In contrast, activated anti-PPD LC could not be detected in the brains of any of 18 rats investigated. Thus, activated LC specific to either BP or PPD were found to accumulate in the thymus, while only activated anti-BP cells accumulated in the brain and caused EAE.

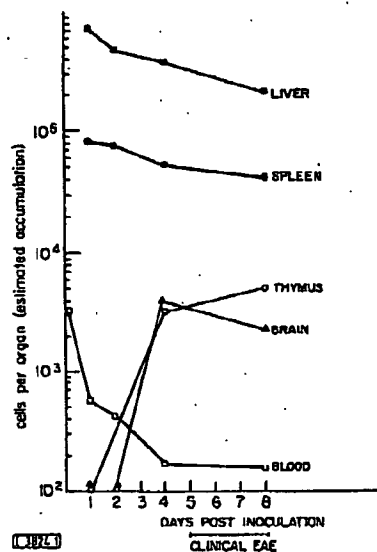


Figure 1. Activated anti-BP LC accumulate in the brain and thymus. Lewis rats were inoculated i.v. with 5×10^6 LC that had been activated and labeled with ^{51}Cr and the numbers of cells accumulating in the blood, liver, spleen, thymus or brain were estimated. Each point represents the mean of 2-4 rats. Standard errors were less than 12% of the mean.

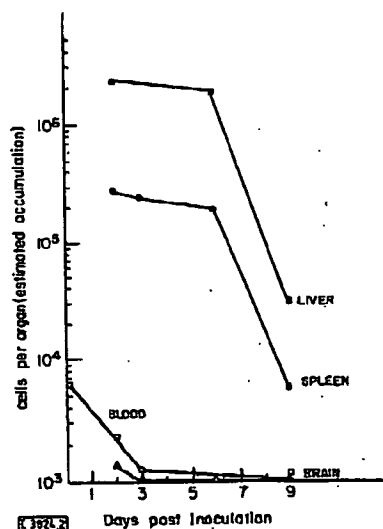


Figure 2. Activated and irradiated anti-BP LC do not accumulate in the brain or thymus. The experiment was done as in Fig. 1, except that the LC were irradiated by 1000 rds before inoculation. "Brain" signifies results for brain or thymus.

To learn whether anti-PPD LC could accumulate in the brain during EAE, we inoculated rats with a mixture of ^{51}Cr -tagged anti-PPD cells and untagged anti-BP cells and estimated the accumulation in the brain of the anti-PPD cells during EAE caused by the anti-BP cells. Table 6 shows that the anti-PPD LC were undetectable in the brains despite the development of EAE in the recipient rats. In contrast, about 13 000 labeled anti-BP LC did accumulate in the brain.

4 Discussion

It was shown earlier that transfer of EAE by lymphocytes obtained from primed animals of several species could be enhanced by incubating the lymphocytes with BP [9-11] or with Con A [12] before inoculation into recipients. The effects of BP or Con A in those transfer experiments could be attributed to an increase in the number or proliferative capacity of the small fraction of anti-BP cells in the heterogeneous population of primed cells. However, the results reported here involving a continuously proliferating population of T lymphocytes, largely, if not exclusively, specific to BP suggest a more fundamental role for activation.

LC maintained in a continuing state of proliferation by culturing them in propagation medium containing IL 2 [13] were incapable, upon i.v. inoculation, of directly mediating EAE (Table 1) or of enhancing resistance to activate EAE (Table 3). However, subsequent to incubation with BP for about 6-8 h, the anti-BP cells acquired the capacity to mediate EAE directly (Table 1). This activation of the LC did not require interaction with specific BP antigen, as incubation with the T cell mitogen Con A was effective (Table 2). These results indicate that the state of the LC, and not the BP antigen itself, was the critical element in realizing the effector potential of the line.

The capability of anti-BP LC to vaccinate against EAE also appeared to require prior activation of the cells *in vitro* (Table 3). As we reported earlier [2], Z1a cells, when attenuated by irradiation or treatment with mitomycin C, did not mediate EAE, but rats receiving such cells acquired resistance to subsequent induction of EAE by injection of BP/CFA. The mechanism of this resistance has yet to be clarified, but it seems to involve some form of immunity to the anti-BP receptors on the T cells [14]. It is possible that activation of these cells *in vitro* might, through changes in the cell membrane, enhance the concentration or exposure of these BP-specific receptors. The receptors on activated LC might then be more accessible and, therefore, more immunogenic when these cells are used as agents of vaccination. The notion that activation might produce changes in membrane receptors was supported by the finding that activation of anti-BP LC was accompanied by acquisition of PNA positivity (Table 4). The finding that activation did not modify the membrane receptors for other lectins suggests that the appearance or increase of the PNA receptors was specific. Moreover, we found that among the population of activated PNA⁺ LC, those that were more readily agglutinable by PNA included the more powerful mediators of EAE (Table 5), suggesting that effector function might be correlated with the concentration or quantity of PNA receptors on activated cells. However, we have no molecular information about the PNA receptors other than that they are situated at the cell membrane and most probably expose a galactose residue [15]. PNA receptors have been associated with the circulatory physiology of lymphocytes [16] and in this regard we found that activated anti-BP LC accumulated in the CNS (central nervous system) and in the thymus, spleen and liver. Activated anti-PPD line cells were not detected in the CNS, although they were found in the other organs tested. Nonactivated or irradiated LC did not accumulate in either the brain or thymus.

The immunospecificity of T lymphocyte line accumulation in the CNS seems to be at variance with the results of earlier investigations involving transfer of labeled cells from the lymphoid organs or donor animals that had been primed with specific antigen. It has been difficult to detect selective accumulation to specific antigen in most [17-20] but not all [21] studies. The majority of cells infiltrating the lesions of EAE and other sites of inflammation were found to be not specifically directed against the particular antigen [19, 20].

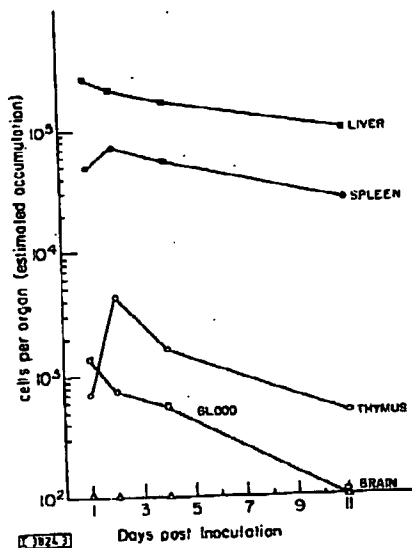


Figure 3. Activated anti-PPD LC accumulate in the thymus but not in the brain. The experiment was done as in Fig. 1.

Table 6. Anti-PPD LC labeled with ^{51}Cr fail to accumulate in the brain during EAE mediated by anti-BP LC

Organ	Anti-BP LC (labeled)	Anti-PPD LC (labeled)
Brain	13,000	0
Thymus	1,000	1,000
Spleen	1,000	1,000
Liver	1,000	1,000

a) Groups of 4 Lewis rats were inoculated i.v. with a mixture of anti-BP (3×10^6) and anti-PPD (3×10^6) LC that were or were not labeled with ^{51}Cr . The accumulation of labeled LC in brains, spleens and livers was estimated 4 days later, at the onset of EAE.

5 References

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However, the apparent lack of specific accumulation in those studies may be explained by the low specific activity of the heterogenous populations of cells transferred from primed donors compared to the exclusive specificity of our LC. On the other hand, our failure to detect nonspecific recruitment of anti-PPD LC in EAE brains could be because the LC had already been differentiated to the point where they were resistant to nonspecific recruitment to inflammatory lesions. In any case, it is evident that autoimmune anti-BP T lymphocytes in the circulation can find the CNS, penetrate it, and mediate disease.

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The accumulation in the thymus of activated anti-BP or anti-PPD LC raises the possibility that the thymus may function in the physiology of fully differentiated T lymphocytes and not serve merely as an organ of early T lymphocyte differentiation [22]. We have been able to recover the progeny of activated anti-BP LC from the thymuses of rats that had been inoculated months earlier with those cells and had recovered from EAE [23]. The recovered cells were able to recognize BP and mediate EAE upon transfer to naive rats. This indicates that anti-BP T lymphocytes, potentially able to produce autoimmune disease, persist in the thymus despite recovery from EAE and acquired resistance to induction of active disease [14]. The role of these persisting cells in immunological memory or in regulation of autoimmunity is a new question raised by these findings.

Another unanswered question is why only a small minority of the anti-BP LC accumulated in the CNS and thymus whereas the vast majority resided in the liver and spleen. It is possible that the latter were injured or abnormal and so were filtered out by scavenger cells of the reticuloendothelial system. However, other explanations are conceivable and perhaps the question can be answered when we succeed in cloning LC and discover how the brain-seeking anti-BP cells differ from the thymus-, liver- and spleen-seekers.

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